Monitoring of Soluble Enzyme Activity by Enzyme Flow Microcalorimetry

V. ŠTEFUCA* and M. POLAKOVIČ

Department of Chemical and Biochemical Engineering, Faculty of Chemical Technology, Slovak University of Technology, SK-812 37 Bratislava
e-mail: stefuca@cvt.stuba.sk

Received 14 June 1999

The possibility of use of the flow microcalorimetry for the enzyme activity testing is demonstrated. Two model enzymes, urease and invertase, were studied, when urea and sucrose were used as substrates. The technique is based on a measurement of temperature change in the sample stream provoked by the reaction heat effect of enzyme reaction. The analysis is conducted so that a pre-cooled sample of enzyme solution is mixed with the substrate solution, and then immediately injected through an injection valve into a carrier buffer pumped continuously into the flow microcalorimeter column packed with glass particles. A temperature change in the column is observed as a heat peak while the peak area depends on the sample volume and on enzymatic reaction rate.

The enzyme activity in the sample was determined from the peak height using a previous microcalorimeter calibration for the known enzyme activity. The set of calibrations was effectuated for both tested enzymes. Enzyme activity ranges of linearity and the influence of substrate concentration on the method sensitivity were investigated. The main advantage of the proposed technique is its versatility due to the versatility of the detection principle.

Enzyme flow microcalorimetry was successfully used for the measurement of the immobilized enzyme activity of different immobilized enzyme systems [1]. The method was based on the experimentally verified assumption about the linear dependence between the thermometric signal and reaction rate. There is, however, always a need of research and industrial people working with enzymes measure regularly the activity of enzyme in its native soluble form. While for one simple measurement many different techniques are acceptable, routine repetitive measurements multiply the time, work and material necessary for the assay, and in order to optimize the laboratory work, one must consider carefully which technique should be used.

An assay of the enzyme activity can be arranged generally in two steps. The first one is the enzyme reaction step. Then, after stopping the reaction, an analytical step is needed for the reactant concentration analysis. These two steps can be effectuated simultaneously when one of the reactants can be monitored directly. The events typically utilized for monitoring of enzyme-catalyzed reactions are the changes in optical properties, either absorption or emission, of the solution or changes of concentration of an ion, most often H^+, detectable electrochemically.

There is a lack of universal methods enabling to monitor a wider range of enzyme reactions. One of possible solutions is calorimetry that can be used for a kinetic investigation of any chemical and enzymatic reactions depending on the reaction enthalpy. The advantages of the kinetic calorimetry are thus obvious. First, the rate of a chemical reaction can be measured without any special requirements being imposed on the reaction medium (solid, viscous, multicomponent systems). Secondly, it is the high efficiency which means a large volume of kinetic information in one experiment and a nondestructive character of changes. Thirdly, the chemical conversion is recorded directly at the time of its occurrence [2].

There are two main experimental configurations of calorimetry used for the determination of enzyme activity – batch [3—7] and flow [8—10]. In the present article, a simple procedure based on the flow injection analysis principle is developed.

EXPERIMENTAL

Urease (Type III from Jack Beans, Sigma, St. Louis, USA, 31 Units/mg solid) and invertase (Grade VII, from bakers' yeast, Sigma, St. Louis, USA, 400 Units/mg solid) were used as model enzymes. All chemicals used as buffer components and enzyme substrates were of anal. grade and provided by Sigma Co.

*The author to whom the correspondence should be addressed.

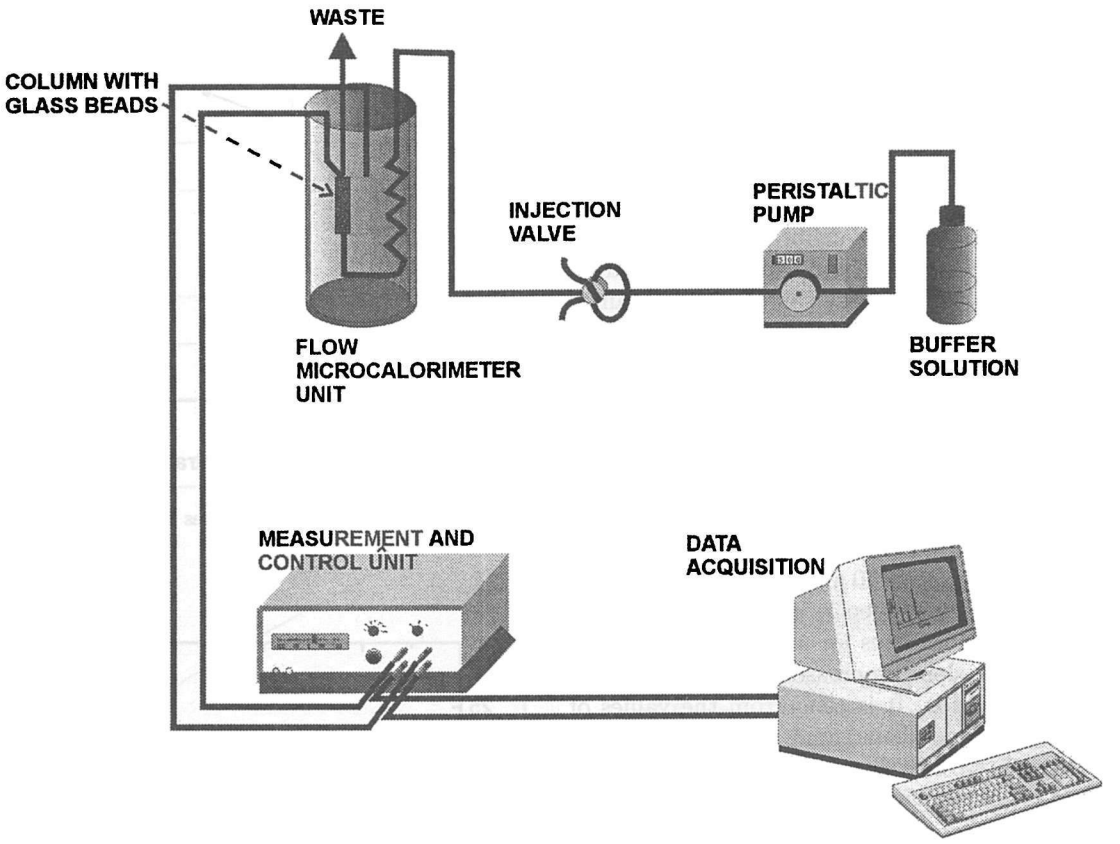

Fig. 1. Experimental set-up for the flow microcalorimetry.

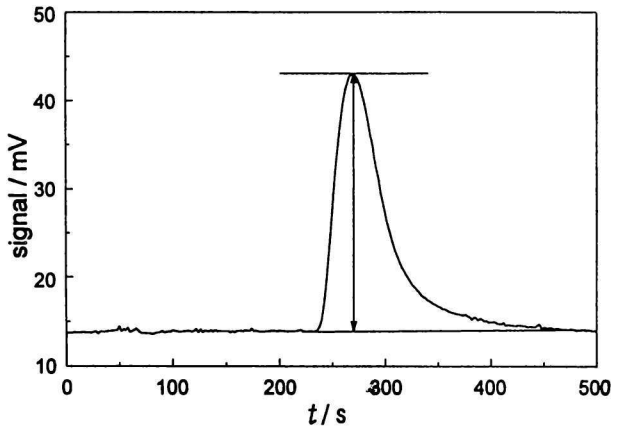

Fig. 2. Example of thermometric registration from a single injection of urea – urease sample.

Enzyme Flow Microcalorimetry

The enzyme flow microcalorimetric measurement is based on the registration of the temperature change provoked by the heat of the reaction catalyzed by an enzyme. The calorimetric measurements were performed using the enzyme flow microcalorimeter (3300 Thermal Assay Probe, Advanced Biosensor Technology AB, Lund, Sweden). The experimental set-up used for the flow injection analysis of enzyme activity is depicted in Fig. 1. The sample of enzyme mixed with the substrate solution is injected through an injection valve into the buffer stream. The enzyme reaction takes place mainly in a small column packed with glass beads (diameter of 0.15 mm). The column is placed in a thermostated block and is used as a minireactor with packed bed having standard dimensions of 2 cm in length and 0.4 cm in inner diameter. The output temperature change is measured by thermistors connected to the Wheatstone bridge, the signal is amplified, and registered by the personal computer. A typical registration from a single assay of urease activity is shown in Fig. 2. As indicated in the figure, the peak height was used as a measure of enzyme activity for the measurement evaluation.

Assay Procedure

The urease activity was measured by mixing 0.2 cm^3 of the enzyme solution in 0.1 mmol dm^{-3} phosphate buffer (pH 7) with 0.2 cm^3 of urea solution in the same buffer and its rapid injecting into the flow microcalorimeter. Both, enzyme and buffer, solutions were pre-cooled at 4 °C prior to mixing and injecting for the calorimetric analysis. The flow rate of the carrier buffer solution was 1 cm^3 min^{-1}, the reaction temperature 30 °C, and the injection loop volume 0.1 cm^3.

RESULTS AND DISCUSSION

In the present work, the possibility of use of the flow microcalorimetry for the soluble enzyme activity measurement is demonstrated. Urease and invertase were studied as the enzyme models. Optimum analytical conditions, such as enzyme and substrate concentration were tested. The calibration dependences of the thermometric signal (in millivolts) *vs.* the total enzyme activity injected by the sample were obtained using varying enzyme activities (Figs. 3 and 5). The experimental results in Fig. 3 show three calibrations for different urea concentrations in the samples. The urea concentrations above the Michaelis constant, K_m, value for urease that is approximately 6 mmol dm^{-3} [11], were used in order to supply a sufficient excess of substrate. Similarly, in the case of invertase 1 mmol dm^{-3} sucrose was used that was also above the K_m value being approximately 40 mmol dm^{-3} for this type of invertase [12]. Independently on the urea concentration, the calibration lines for urease were perfectly linear, while their slopes increased with the increasing urea concentration. It is clear from the values of slopes in Table 1 and their trend depicted in Fig. 4 that the increase of urea concentration from 0.5 to 1 mmol dm^{-3} is not significantly beneficial for the analysis sensitivity. Using similar analysis it was found, in the case of invertase, that 1 mmol dm^{-3} of sucrose was sufficiently high concentration for maximum analysis sensitivity. In contrast to urease, a nonlinear course of calibration line was observed (Fig. 6) above certain experimental concentration of invertase. It was probably caused by a substrate depletion due to the substantially high enzyme concentration. The K_m value of invertase is, moreover, nearly seven times higher than that of urease. Therefore, the apparent reaction kinetics for invertase will shift from the zero order with the substrate depletion (this effect is even amplified by product inhibition) more significantly than that for urease.

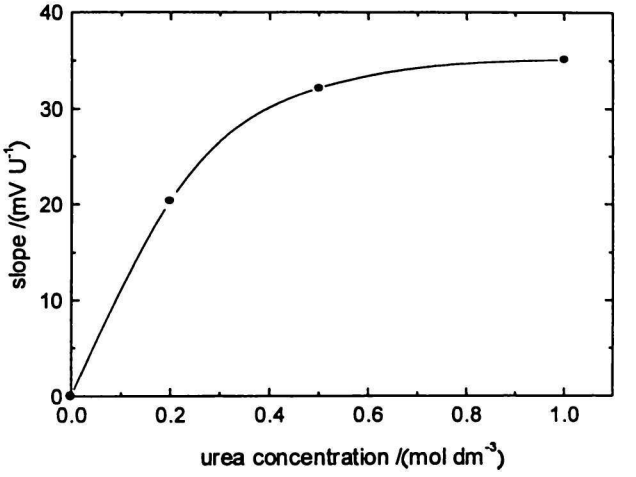

Fig. 4. Slopes of calibration lines for urease *vs.* urea concentration.

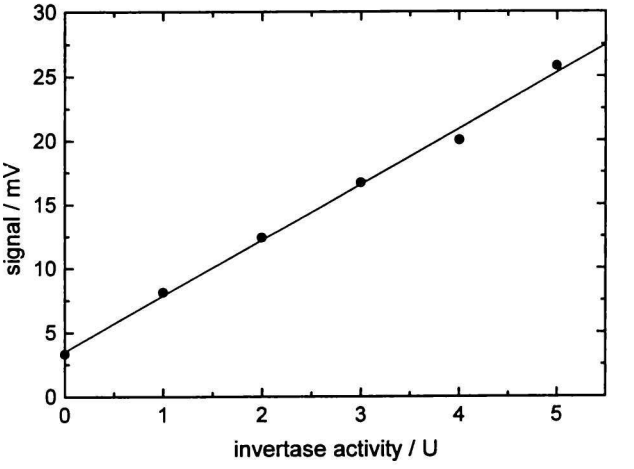

Fig. 5. Calibration results for invertase.

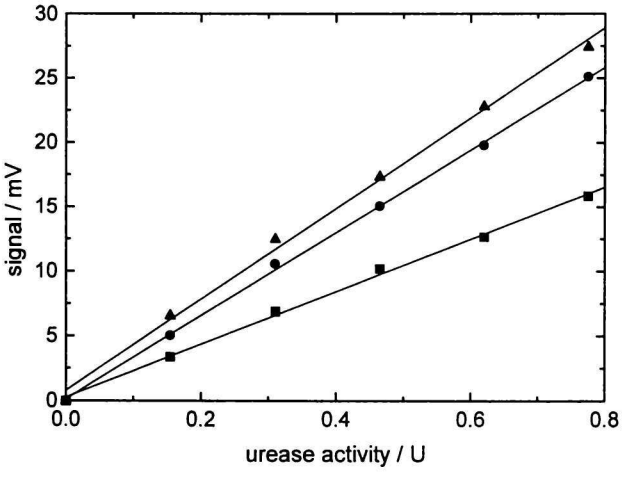

Fig. 3. Calibration results for urease. Urea concentrations (in mmol dm^{-3}): ■ 0.2; ● 0.5; ▲ 1.

When intercepts of calibration straight lines are concerned, the value obtained for invertase is not negligible compared to urease. A certain signal was in fact observed at the injections of blank samples (samples without the enzyme) provoked probably by the mixing heat. Therefore, this effect must be taken into account for a routine analysis.

The comparison of slopes in Table 1 leads to the conclusion that the urease assay is much more sensitive. This is in agreement with the molar reaction enthalpies of urease (61 kJ mol^{-1} [13]) and invertase (14.5 kJ mol^{-1} [14]) catalyzed reactions.

CONCLUSION

Experimental conditions for the analyses of activ-

Table 1. Linear Regression Data of Microcalorimeter Calibration for Urease and Invertase

Substrate concentration	Slope (error)	Intercept (error)	Correlation coefficient
mmol dm^{-3}	mV U^{-1}	mV	
	Urease		
0.2	20.35 (0.53)	0.27 (0.24)	0.9987
0.5	32.13 (0.43)	0.13 (0.20)	0.9996
1.0	35.10 (1.02)	0.79 (0.47)	0.9983
	Invertase		
1.0	4.00 (0.12)	5.68 (0.37)	0.9987

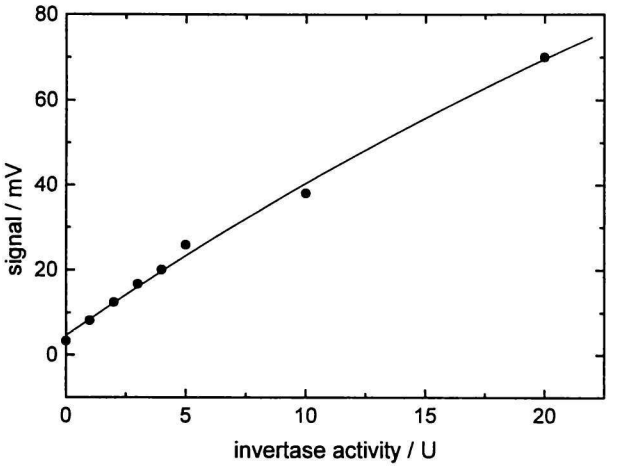

Fig. 6. Calibration results for invertase – broader range of invertase activity.

ities of urease and invertase by the injection flow microcalorimetry were determined by this preliminary work. This technique could be useful in the cases when a rapid and simple measurement is required, *e.g.* during enzyme purification, for effluent enzyme activity monitoring during chromatographic separation, in inactivation studies, in screening of enzymes. The presented technique has been used for inactivation studies of invertase and urease. The goal of the future study is to determine a more general strategy of the optimization of equipment configuration and conditions of analysis regarding to maximum analysis sensitivity. This will be achieved using the mathematical modelling based on mass and heat balances.

Acknowledgements. The work was partially financed by the Slovak Grant Agency for Science (grant VEGA 2/4149/97).

REFERENCES

1. Štefuca, V. and Gemeiner, P., in *Advances in Biochemical Engineering/Biotechnology,* Vol. *64.* (Scheper, T., Editor.) P. 71. Springer-Verlag, Berlin, 1999.
2. Barkalov, I. M. and Kiryukhin, D. P., *Int. Rev. Phys. Chem. 13,* 337 (1994).
3. Grime, J. K., Tan, B., *et al., Anal. Chim. Acta 109,* 393 (1979).
4. Kurvits, M. and Siimer, E., *Thermochim. Acta 103,* 297 (1986).
5. Williams, B. A. and Toone, E. J., *J. Org. Chem. 58,* 3510 (1993).
6. Liang, Y., Wang, C. X., *et al., Thermochim. Acta 268,* 17 (1995).
7. Salieri, G., Vinci, G., *et al., Anal. Chim. Acta 300,* 287 (1995).
8. Danielsson, B. and Mossbach, K., *FEBS Lett. 101,* 47 (1979).
9. Danielsson, B. and Mossbach, K., *Methods Enzymol. 137,* 181 (1988).
10. Beran, M. and Paulíček, V., *J. Therm. Anal. 38,* 1979 (1992).
11. Kohutovičová, E., *Diploma Thesis.* Slovak University of Technology, Bratislava, 1998.
12. Štefuca, V., Gemeiner, P., and Báleš, V., *Enzyme Microb. Technol. 306,* 10 (1988).
13. Owusu, R. K., Trevhella, M. J., and Finch, A., *Thermochim. Acta 111,* 1 (1987).
14. Goldberg, R. N., Tewari, Y. B., and Ahluwalia, J. C., *J. Biol. Chem. 264,* 9901 (1989).